

Evaluating the use of DNA Sequences for Species Identification in Medusozoans (Phylum Cnidaria)

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INTRODUCTION

The Phylum Cnidaria is comprised of two subphyla: Anthozoa and Medusozoa. Anthozoa are sessile polyps lacking a medusa stage in their life cycle. Medusozoa, which comprise almost 4000 species (Daly et al., 2007) can have a free-swimming medusa stage and a benthic polyp stage as part of their life-cycle. However there are many modifications of this life cycle in different species of medusozoans (Brusca and Brusca, 2003). The medusozoans are also unique in that they have a linear mitochondrial genome (Ortman et al., 2010). Medusozoa is divided into four separated classes: Hydrozoa, Cubozoa, Scyphozoa, and Staurozoa. Hydrozoans are the most diverse class of medusozoans (approximately 3,500 species) and comprise the groups: Filifera, Capitata, Aplanulata, and Leptothecata, Siphonophora, and Trachylina (Daly et al., 2007). Due to the variety of morphologies, life-cycles, and sheer number of species, understanding medusozoan diversity can be enigmatic and therefore interesting to study.

Identifying species based on DNA sequences, often referred to as DNA barcoding, has a variety of practical and theoretical applications. This technique can be used to identify endangered species targeted for poaching or illegal importing and exporting (Dalton and Kotze, 2010) as well as disease tracking (Alcaide et al., 2009), discovery of new

species (Meyer and Paulay, 2005), and to reveal cryptic species (C.J. Moura et al., 2011). More generally, DNA barcoding can be used to quickly identify species by comparing a sequence from an unknown species to an existing database of DNA sequences from species that have been properly identified from a taxonomic expert and vouchered through a museum specimen. Once this database has been developed, further taxonomic expertise would not be required to identify a species through a DNA sequence. In other words, these DNA barcodes are auxiliary characters that allow identification of an unknown specimen in terms of a known classification (Bucklin et al., 2010). DNA barcoding also elucidates the possibility to test taxonomic hypotheses and provide new insights into evolution (C.J. Moura et al., 2011). This is especially relevant in organisms such as jellyfish, that can be easily damaged during collection; making species identification by subtle morphological traits impossible (Ortman et al., 2010). Although DNA barcoding has many applications, more investigations are needed to evaluate the ability of a relatively short DNA sequence to distinguish between species. Identifying a DNA barcoding molecule that efficiently and correctly categorizes species would allow for a relatively low cost way to identify species, where taxonomic expertise is lacking or the specimen is not preserved in a way to identify based on morphology alone.

Currently there are efforts to use molecular barcoding as the standard of identification of biological species. This initiative, called Barcode of Life, intends to identify all possible multicellular species into an online database (<http://www.barcodeoflife.org>). The standard molecular barcoding molecule used for this project is CO1 (Hebert et al., 2003). An ideal barcoding marker should have low intra-specific and a relatively higher inter-specific sequence variation (Meyer and Paulay, 2005). It is also necessary that variation within species is less than – and does not overlap with – variation between species (Ortman et al., 2010). This gap between genetic distances of intra-specific variation and inter-specific variation is termed the “barcoding gap”. The larger the barcoding gap, the better the molecule at delimiting species. It has been suggested that CO1 is not the best barcoding molecule for all organisms (Hollingsworth et al., 2009; Vences et al., 2005; Shearer et al., 2008) and due to the variable rate of mutation between different groups of organisms, more research is needed before concluding that CO1 can be used as the standard molecular barcoding molecule for all multicellular life.

In plants, CO1 has a lower substitution rate of mitochondrial DNA and it has been seen that plastid genes are more efficient (Hollingsworth et al., 2009). CO1 is also inappropriate to use in the sister group of medusozoans, anthozoans, due to the slow evolutionary rate in the CO1 gene (Shearer et al., 2008). The overlap between intraspecific variation and interspecific divergence results in the inability to establish an appropriate threshold value for anthozoans (Shearer et al., 2008). In amphibians, CO1 exhibits a high rate of

variation both at the level of groups and similar species (Vences et al., 2005). Not only is CO1 unsuitable for use in amphibians, it is suggested that the 16S rDNA gene is better at delimiting between species.

For medusozoans, a study done by Ortman et al. suggests CO1 is sufficient and indicative of species delimitation for Scyphozoa and Hydrozoa. Due to a faster rate of evolution in the mitochondria, CO1 exhibits favorable levels of divergence within and between species. This allows CO1 to be seen as an indicator of speciation. The other barcoding molecule that is useful among medusozoans is the mitochondrial large ribosomal subunit (16S) rDNA gene. 16S has been found to be much easier to amplify than CO1, particularly in the hydrozoan clades Anthoathecata and Leptothecata groups (C.J. Moura et al., 2011). In addition, 16S genetic information of hydrozoans has been found to be consistent with nuclear genetic markers and morphology (C.J. Moura et al., 2011). This is extended evidence for the potential of 16S as a barcoding molecule. It has allowed for recognition and discrimination of nominal and cryptic hydrozoan species (C.J. Moura et al., 2011). In medusozoans, it has been sequenced extensively and is readily available in public databases.

We generated CO1 and 16S DNA sequences from medusozoan species and assembled large datasets of both CO1 and 16S sequences from newly generated and publicly available sequences. We used these large datasets to compare multiple groups of medusozoans including Cubozoa, Staurozoa, Scyphozoa, and the hydrozoan groups, Filifera, Aplanulata, Siphonophora, Leptothecata, Trachylina, and Capitata. We analyzed both CO1 and

16S to determine their utility to delimit species and thus to be used as a DNA barcoding molecule.

MATERIALS AND METHODS

DNA used for this study was from an existing collection provided by Paulyn Cartwright. PCR amplification was performed using the primers 16S: F2 ('TCGACTGTTTACCAAAAACATAGC') and R2 ('ACGGAATGAACTCAAATCATGTAAG') (Cunningham and Buss, 1993); 16S: SHB ('GACTGTTTACCAAAAACATA') and BR ('CATAATTCAACATCGAGG') (Cunningham and Buss, 1993; Schroth et al., 2002); CO1: F1 ('GGTCAACAAATCATAAAGATATTGG') and R1 ('TAAACTTCAGGGTGACCAAAAAATCA') (Folmer et al., 1994); and CO1: HCOcato ('CCTCCAGCAGGATCAAAGAAAG') and LCOjf ('GGTCAACAAATCATAAAGATATTGGAAC') (Dawson, 2005). PCR conditions for primers CO1: HCOcato and LCOjf used a ramp-up program. The steps for this program are as follows: 1) 94°F for 8 minutes 2) 49°F for 2 minutes 3) 72°F for 2 minutes 4) 94°F for 4 minutes 5) 50°F for 2 minutes 6) 72°F for 2 minutes 7) 94°F for 45 seconds 8) 51°F for 45 seconds 9) 72°F for 1 minute 10) go to step 7, 32 times 11) 72°F for 10 minutes. For all other primers we used a different ramp-up program. The steps for this program are as follows: 1) 94°F for 5 minutes 2) 94°F for 50 seconds 3) 45°F for 50 seconds 4) 72°F for 1 minute 5) go to step 2, 4 times 6) 94°F for 50 seconds 7) 50°F 1 minute 8) 72°F 1 minute 9) go to step 7, 29 times 10) 72°F for 5 minutes. PCR fragments were evaluated by size using gel electrophoresis. Unpurified PCR products were plated and sent to the

University of Washington DNA sequencing center for sequencing.

Sequences were edited and assembled in the software program Geneious (Drummond et al., 2011). DNA sequences were aligned using the algorithm Muscle (Edgar, 2004) in the program SeaView (Gouy et al., 2010). Pairwise distances were calculated using the Kimura 2-Parameter (K2P) and genetic distances were plotted using the R (R Development Core Team, 2012) package SPIDER (Brown et al., 2012). Graphs plot the genetic distances on the x-axis and the density on the y-axis. The density plot represents the amount of sequences, at a particular genetic distance, as a percentage of all the available sequences. Pairwise genetic distances were calculated between all CO1 and 16S sequences within a species to determine intraspecific variation. To determine interspecific variation, pairwise genetic distances were calculated between species for all groups except CO1 sequences in Cubozoa, where data was only available for a single species.

RESULTS

We generated DNA sequences for 16S and CO1 and combined them with publicly available sequences. Our combined database consisted of 864 16S sequences and 765 CO1 sequences from a variety of medusozoan species. There were 60 Capitata, 52 Filifera, 156 Scyphozoa, 219 Aplanulata, 69 Siphonophora, 21 Cubozoa, 153 Leptothecata, 26 Trachylina, and 4 Staurozoa DNA samples that were barcoded using CO1. There were 97 Capitata, 204 Filifera, 63 Scyphozoa, 47 Aplanulata, 49 Siphonophora, 57 Cubozoa, 257 Leptothecata, 63

Trachylina, and 21 Staurozoa DNA samples that were barcoded using 16S. A list of all species sampled, Genbank sequences numbers when available, and newly generated sequences are found in Supplemental Table 1.

Through plotting the density of genetic distances of pairwise comparisons of genetic variation within and between species of different groups of medusozoans, we can determine if the genetic variation within species is less than the genetic variation between species. If there is no overlap between intra- and inter-specific variation, we can conclude that there is a barcoding gap and the molecule can serve to delimit species of every pairwise comparison. We created graphs of density plots of genetic distances of pairwise sequence comparison within and between species in the different groups of medusozoans for 16S and CO1 sequences (Figure 1 and Figure 2).

Figure 1 depicts intra- and inter-specific genetic variation in the medusozoan classes Staurozoa, Cubozoa, Scyphozoa and in the hydrozoan subclass Trachylina. In Staurozoa a barcoding gap exists in CO1, in that there is greater and non-overlapping genetic distance between intra- than interspecific variation. There was no barcoding gap in 16S sequences of Staurozoa in that there was substantial overlap in the intra- and inter-specific variation and the inter-specific variation was very wide ranging. For Cubozoa, CO1 sequence data was not available for more than one species, so this analysis could not be done. For 16S sequences in Cubozoa, there was substantial overlap in the intra- and inter-specific variation and inter-specific variation was very wide-ranging. In Scyphozoa, the CO1 data reveals two distinct peaks distinguishing intra- and

inter-specific variation, whereas there was substantial intra-specific variation in 16S, overlapping with the inter-specific variation. In the hydrozoan subclass Trachylina, both molecules have significant intra-specific variation that exceeds that of inter-specific variation.

Figure 2 depicts intra- and inter-specific genetic variation in the different groups of the hydrozoan subclass Hydroidolina: Leptothecata, Filifera, Siphonophora, Capitata and Aplanulata. In CO1 comparisons of Leptothecata sequences, there are two distinct peaks distinguishing intra- and interspecific variation, with some pairwise comparisons falling outside these peaks for both the intra- and interspecific variation. The 16S data shows two distinct peaks with substantial overlap, and 16S also has an additional peak of intra-specific variation that surpasses that of inter-specific. In Filifera, CO1 and 16S have significant intra-specific variation whose range exceeds that of inter-specific variation, although the peak of intraspecific variation is less than the peak of interspecific variation. While both molecules display distinct peaks of genetic variation, there substantial overlap between intra- and interspecific variation. In Siphonophora CO1 and 16S comparisons, we see that the inter-specific variation spreads across the entire graph, overlaying with intra-specific variation. In Capitata, CO1 reveals two distinct peaks distinguishing intra- and interspecific variation with additional small peaks of highly variable intra- and inter-specific variation. Although 16S comparisons in Capitata display distinct peaks, there is considerably more overlap in intra- and inter-specific variation than displayed in CO1. In Aplanulata, the CO1 molecule displays intra-specific variation that almost completely surpasses that of

the inter-specific variation – the opposite of what is expected. 16S produces a variable amount of intra-specific variation, but distinct peaks are still present.

We estimated the average percent overlap, where intra-specific variation overlapped with inter-specific variation, for each graph (see Table 1). We then averaged the total overlap for each barcoding molecule. CO1 has a lower

overlap on average, than 16S. We then performed a Two-Sample Student-t Test of the average means and found: T-Value - 1.54, P-Value 0.148, DF=13. For CO1 the StDev=22.4 and SE Mean=7.9. For 16S the StDev=17.2 and SE Mean=6.1. Therefore there is no significant difference between 16S and CO1 in general as a barcoding molecule for average percent overlap.

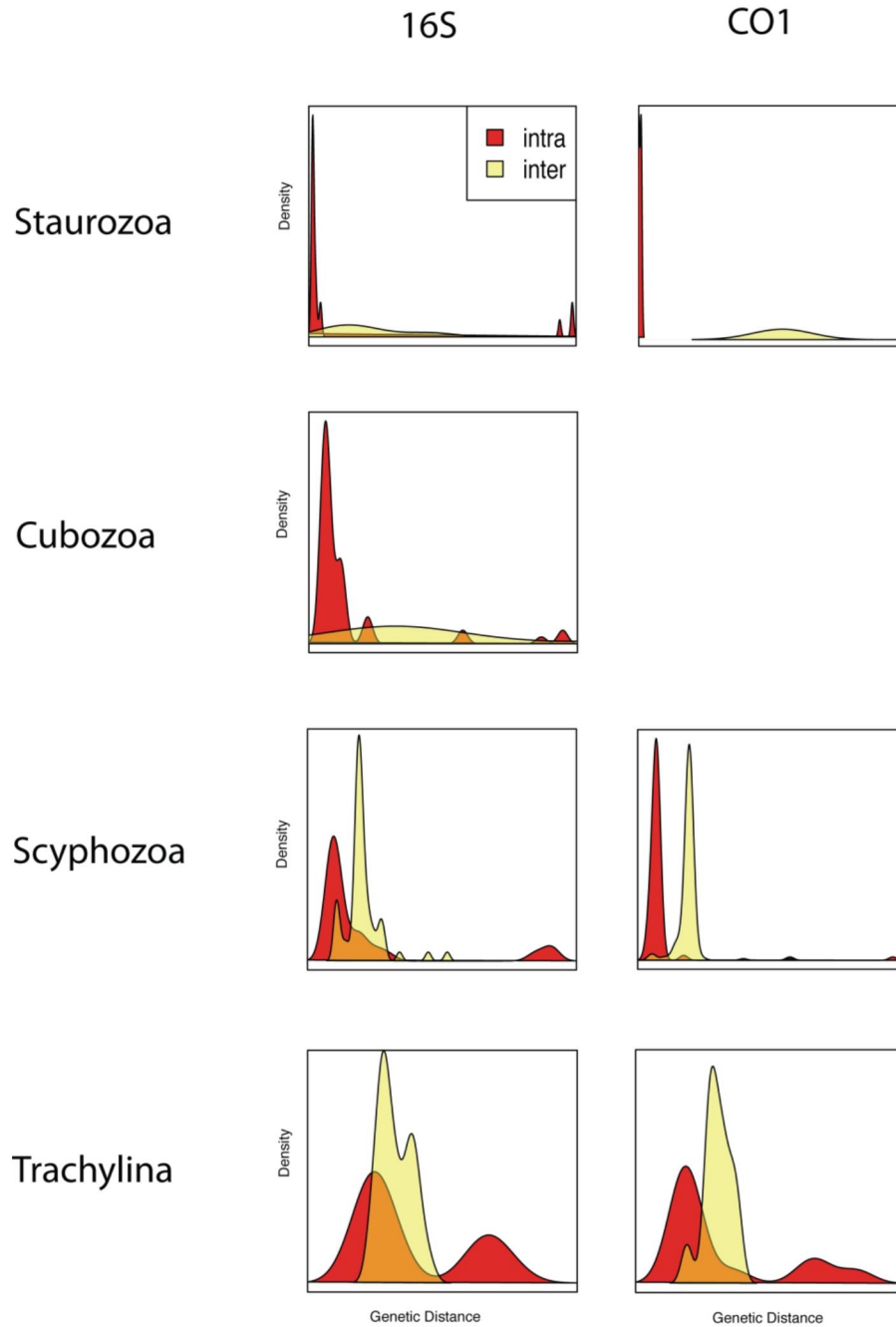


Figure 1. Density plots of pairwise comparisons of genetic variation of 16S and CO1 sequences for the medusozoan classes Staurozoa, Cubozoa, Scyphozoa and in the hydrozoan subclass Trachylina. The x-axis is “genetic distance” or genetic variation for pairwise comparisons. The y-axis is density of genetic distances of pairwise comparisons. Red indicates the amount of intra-specific variation and the yellow signifies the amount of inter-specific variation.

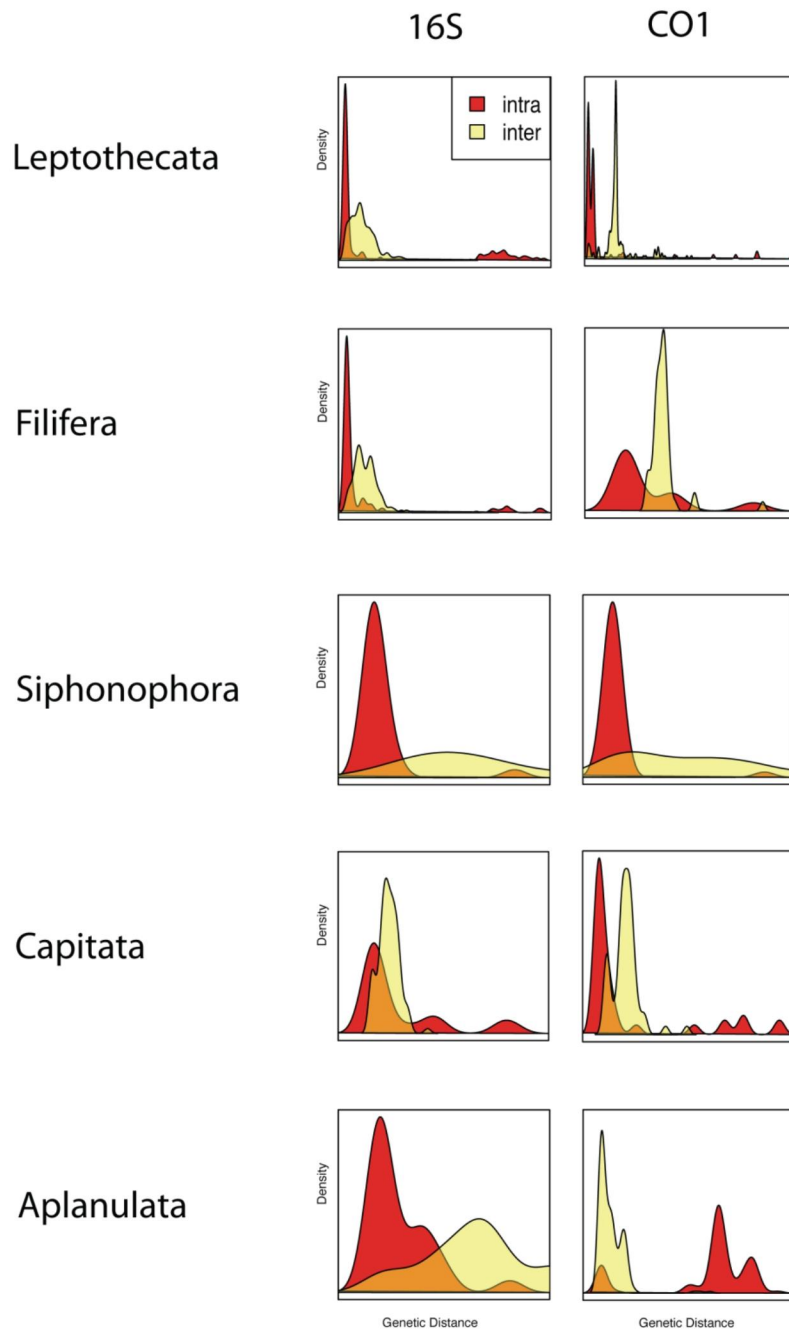


Figure 2. Density plots of pairwise comparisons of genetic variation of 16S and CO1 sequences for the Leptothecata, Filifera, Siphonophora, Capitata and Aplanulata, which are groups within the hydrozoan subclass Hydroidolina. The y-axis is density of genetic distances of pairwise comparisons. Red indicates the amount of intra-specific variation and the yellow signifies the amount of inter-specific variation.

Table 1. Percent overlap for each barcoding molecule in each group of organisms. The percent overlap is the total amount of intra-specific variation that overlays with the inter-specific variation. We averaged over all groups to find an average percent overlap for each barcoding molecule.

Group	CO1 % Overlap	16S % Overlap
Trachylina	20.93	40.00
Staurozoa	0	44.83
Leptothecata	10.07	46.86
Scyphozoa	7.22	31.25
Aplanulata	8.96	16.05
Capitata	71.43	43.70
Filifera	10.64	49.45
Siphonophora	1.03	21.05
AVERAGE	32.30	18.79

DISCUSSION

A perfect barcoding molecule would produce low intra-specific variation and higher inter-specific variation and no overlap between these two distributions (Meyer and Pauley, 2005). However, these seamless results are rarely seen, but can often lead us to interesting conclusions. The lack of a barcoding gap can help us to elucidate changes in an organism's evolutionary history, expose error in identification and suggest new classifications.

In Staurozoans, analyses of CO1 sequences shows that it displays a perfect barcoding gap (see Figure 1). However, this could be due to the low sampling size ($n=4$). Similarly, for 16S in Staurozoans, the substantial overlap in pairwise comparisons and wide-ranging inter-specific variation could simply be due to a lower sampling size ($n=21$). However, because an overlap also indicates that the barcoding molecule cannot distinguish if two organisms are of the same species or of different species groups, the large overlap could indicate that the rate of sequence evolution is not rapid enough to distinguish a more recent speciation event from two genetically distinct individuals of the same species. An ideal barcoding molecule should be able to distinguish closely related species for individuals of the same species. If the molecule cannot separate two different species, it is possible the molecule's sequence did not mutate at a fast enough rate to distinguish recent speciation events.

The same is true for cubozoans in analyses of 16S sequences. The inter-specific variation crosses the entire span of the graph, indicating that the group may have speciated over a large period of

time, with some pairwise comparisons between more distantly related species exhibiting large genetic divergences whereas others more closely related species exhibiting low genetic divergence (see Figure 1). Also, there are tiny peaks of intra-specific variation that are very variable. This could mean that there are incorrectly identified species in Genbank, leading the graph to portray a wide intra-specific variation incorrectly.

In Scyphozoans, analyses of the CO1 barcoding molecule shows very little overlap between intra- and inter-specific variations (see Figure 1). Both intra-specific and inter-specific variations are in peaks, meaning that the majority of intra-specific variation is less than the majority of inter-specific variation. In 16S, a small peak of intra-specific variation that surpasses the inter-specific variation is likely due to misidentification.

In analyses of both 16S and CO1 in Trachylina, an interesting trend appeared where a very significant peak in intra-specific variation surpassed inter-specific variation. Although misidentification could explain this pattern, it also suggests that there are genetic distinct lineages within Trachylina that are currently designated within the same species (see Figure 1). The bimodal distribution is evident more drastically in 16S with a higher peak than in CO1. There is also significant overlap in both sequences as well, adding evidence to potential recent speciation events. These data suggest that further taxonomic investigations regarding species delimitations in Trachylina are warranted.

In Leptothecata, analyses of CO1 sequences demonstrate that it yields a clear barcoding gap. There is minimal overlap and what overlaps are tiny peaks

that likely represent misidentification. On the other hand, the 16S barcoding molecule expresses a clear overlap and high intra-specific variation. Within intra-specific variation we can see two distinct groupings, possibly indicating misidentifications and species delimitation issues that need to be further studied.

Analyses of the 16S and CO1 barcoding molecules both display a lack of a barcoding gap in Filifera. In 16S there is some intra-specific variation that surpasses the inter-specific variation, but it is insignificant enough to be considered misidentification. The complete overlap also indicates the molecule's inability to distinguish between and within species. CO1 produces a lot of variable intra-specific variation. It is possible that CO1 is indicating misidentifications.

The patterns of intra- and inter-specific variation in Siphonophora are almost exactly the same in analyses of CO1 and 16S sequences. A peak of intra-specific variation shows that the majority of intra-specific data is less than inter-specific data. There is a small peak in both graphs of intra-specific variation that surpasses the majority of inter-specific variation. This indicates possible misidentification. The wide ranging inter-specific variation is likely evidence that Siphonophora speciated over a large period of time with more closely related species displaying genetic variation less or equivalent intra-specific variation, whereas more distantly related species display significant genetic variation.

For Capitata, analyses of the 16S barcoding molecule, shows that there is no barcoding gap. The three separate peaks of intra-specific variation likely represent misidentification and/or undetected speciation events, sometimes called cryptic speciation. The complete

overlap of intra- and inter-specific variation elucidates the inability of the 16S sequence to differentiate among and within species. The CO1 sequence produces two distinct peaks between intra- and inter-specific variations. The small peaks of intra-specific variation likely represent misidentification of species.

In Aplanulata, analyses of 16S sequences show two distinct peaks of intra- and inter-specific variation, however, there is still a large amount of overlap. The peaks simply mean that the majority of intra-specific variation is less than the majority of inter-specific variation. The wide-ranging inter-specific variation could mean that Aplanulata speciated over a long period of time and the rate of sequence evolution of 16S is not rapid enough to distinguish more recent speciation events. Using the CO1 sequence, we found an interesting phenomenon where the intra-specific variation completely surpasses that of inter-specific variation, creating a false barcoding gap. Within the group Aplanulata are many issues with species delimitation. For example, the genus *Hydra* contains cosmopolitan species. Molecular phylogenetic analyses have shown that many *Hydra* species actually contain several distinct lineages and most currently described species are polyphyletic (i.e. members of the species do not share a single common ancestor (Martinez et al., 2010). Given these issues with species limitation, the intra-specific analyses actually contain a substantial amount of actual inter-specific comparisons, confounding the analyses.

Based on our results, we find CO1 appears to have been a better barcoding molecule overall, producing more distinctive peaks and less average overlap between intra- and inter-specific

variation than 16S. Analyses on a group-by-group basis revealed many interesting issues that warrant further study. Many of our analyses suggested that there might be a significant number of misidentifications in the Genbank sequences. In addition, some of our analyses implied that there are issues with species delimitation and that there are likely unrecognized distinct species that are in need of description and taxonomic revision. Lastly, while barcoding molecules may be helpful in matching sequences to a database of sequences from specimens which have been properly identified, care should be taken when using percent divergence between either CO1 or 16S to determine species delimitations, as there is generally substantial overlap between intra- and inter-specific genetic divergences and no clear barcoding gap exists.

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